



## Genotype-outcome correlations in pediatric AML: the impact of a monosomal karyotype in trial AML-BFM 2004

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**Abstract:** We conducted a cytogenetic analysis of 642 children with de novo acute myeloid leukemia (AML) treated on the AML-Berlin-Frankfurt-Münster (BFM) 04 protocol to determine the prognostic value of specific chromosomal aberrations including monosomal (MK+), complex (CK+) and hypodiploid (HK+) karyotypes, individually and in combination. Multivariate regression analysis identified in particular MK+ (n=22) as a new independent risk factor for poor event-free survival (EFS  $23\pm 9\%$  vs  $53\pm 2\%$  for all other patients,  $P=0.0003$ ), even after exclusion of four patients with monosomy 7 (EFS  $28\pm 11\%$ ,  $P=0.0081$ ). CK+ patients without MK had a better prognosis (n=47, EFS  $47\pm 8\%$ ,  $P=0.46$ ) than those with MK+ (n=12, EFS  $25\pm 13\%$ ,  $P=0.024$ ). HK+ (n=37, EFS  $44\pm 8\%$  for total cohort,  $P=0.3$ ) influenced outcome only when t(8;21) patients were excluded (remaining n=16, EFS  $9\pm 8\%$ ,  $P<0.0001$ ). An extremely poor outcome was observed for MK+/HK+ patients (n=10, EFS  $10\pm 10\%$ ,  $P<0.0001$ ). Finally, isolated trisomy 8 was also associated with low EFS (n=16, EFS  $25\pm 11\%$ ,  $P=0.0091$ ). In conclusion, monosomal karyotype is a strong and independent predictor for high-risk pediatric AML. In addition, isolated trisomy 8 and hypodiploidy without t(8;21) coincide with dismal outcome. These results have important implications for risk stratification and should be further validated in independent pediatric cohorts.

DOI: <https://doi.org/10.1038/leu.2017.121>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-149063>

Journal Article

Published Version



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Originally published at:

Rasche, M; von Neuhoff, C; Dworzak, M; Bourquin, J-P; Bradtke, J; Göhring, G; Escherich, G; Fleischhack, G; Graf, N; Gruhn, B; Haas, O A; Klingebiel, T; Kremens, B; Lehrnbecher, T; von Stackelberg, A; Tchinda, J; Zemanova, Z; Thiede, C; von Neuhoff, N; Zimmermann, M; Creutzig, U; Reinhardt, D

(2017). Genotype-outcome correlations in pediatric AML: the impact of a monosomal karyotype in trial AML-BFM 2004. *Leukemia*, 31(12):2807-2814.  
DOI: <https://doi.org/10.1038/leu.2017.121>

## ORIGINAL ARTICLE

## Genotype-outcome correlations in pediatric AML: the impact of a monosomal karyotype in trial AML-BFM 2004

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We conducted a cytogenetic analysis of 642 children with *de novo* acute myeloid leukemia (AML) treated on the AML-Berlin-Frankfurt-Münster (BFM) 04 protocol to determine the prognostic value of specific chromosomal aberrations including monosomal (MK<sup>+</sup>), complex (CK<sup>+</sup>) and hypodiploid (HK<sup>+</sup>) karyotypes, individually and in combination. Multivariate regression analysis identified in particular MK<sup>+</sup> ( $n=22$ ) as a new independent risk factor for poor event-free survival (EFS  $23 \pm 9\%$  vs  $53 \pm 2\%$  for all other patients,  $P=0.0003$ ), even after exclusion of four patients with monosomy 7 (EFS  $28 \pm 11\%$ ,  $P=0.0081$ ). CK<sup>+</sup> patients without MK had a better prognosis ( $n=47$ , EFS  $47 \pm 8\%$ ,  $P=0.46$ ) than those with MK<sup>+</sup> ( $n=12$ , EFS  $25 \pm 13\%$ ,  $P=0.024$ ). HK<sup>+</sup> ( $n=37$ , EFS  $44 \pm 8\%$  for total cohort,  $P=0.3$ ) influenced outcome only when t(8;21) patients were excluded (remaining  $n=16$ , EFS  $9 \pm 8\%$ ,  $P<0.0001$ ). An extremely poor outcome was observed for MK<sup>+</sup>/HK<sup>+</sup> patients ( $n=10$ , EFS  $10 \pm 10\%$ ,  $P<0.0001$ ). Finally, isolated trisomy 8 was also associated with low EFS ( $n=16$ , EFS  $25 \pm 11\%$ ,  $P=0.0091$ ). In conclusion, monosomal karyotype is a strong and independent predictor for high-risk pediatric AML. In addition, isolated trisomy 8 and hypodiploidy without t(8;21) coincide with dismal outcome. These results have important implications for risk stratification and should be further validated in independent pediatric cohorts.

Leukemia (2017) 31, 2807–2814; doi:10.1038/leu.2017.121

## INTRODUCTION

In recent years, analyses of molecular and cytogenetic aberrations have revealed the heterogeneity of pediatric acute myeloid leukemia (AML),<sup>1,2</sup> which is now partially incorporated within the World Health Organization classification and current risk stratification systems.<sup>3–5</sup> To date, most study groups have agreed on favorable prognostic factors such as inv(16)(p13.1;q22)/CBFB-MYH11, or t(16;16)(p13.1;q22), t(8;21)(q22;q22)/RUNX1-RUNX1T1, t(15;17)(pML-RARA, single *NPM1* mutations or double mutated *CEBPA*.<sup>1,2,4–6</sup> However, conflicting data on risk factors for poor outcomes represent the highly variable definitions of high-risk AML among international study groups and reflect the urgent need to analyze potentially high-risk aberrations in large pediatric cohorts.

Monosomy 7 is a well-described unfavorable prognostic factor in pediatric and adult patients with AML.<sup>2,7</sup> In adult AML, a defined group of patients harboring a monosomal karyotype (MK) has been found to experience exceedingly poor outcomes.<sup>8–13</sup> However, in children, the predictive relevance of a MK remains unclear.<sup>14</sup> A recent study elucidated the impact of modal numbers in pediatric AML, assuming that a hypodiploid karyotype (HK) may

be related to poor outcome.<sup>15</sup> For complex karyotype (CK), however, varying definitions exist and patients are treated heterogeneously. Even with differences in karyotype definitions, current collaborative studies mostly recommend allogeneic stem cell transplantation (HSCT) during the first complete remission (1st CR) for treatment of genetically defined high-risk patients.<sup>16</sup> Thus, it is crucial to carefully define high-risk factors.

In this study, we evaluated correlations between genotype and outcome for defined aberrant karyotypes in a large, uniquely treated group of children with AML in the AML-Berlin-Frankfurt-Münster (BFM) 04 study and considered the results within the context of the increasing complexity of genetic aberrations.

## PATIENTS AND METHODS

## Patients

Between March 2004 and March 2012, 764 patients 0–18 years of age with *de novo* AML in Germany, Austria, Switzerland, and the Czech Republic (patients with Down Syndrome excluded) were treated according to the AML-BFM 04 protocol (ClinicalTrials.gov Identifier: NCT00111345). Initial diagnosis was performed according to the French-American-British (FAB)

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Presented in part at the 47th Congress of the International Society of Paediatric Oncology, 8–11 October, 2015, Cape Town, South Africa and at Acute Leukemias XV 22–25 February, 2015, Munich and at the 21st Congress of European Hematology Association, Copenhagen, 9–12 June, 2016, Copenhagen.

Received 5 September 2016; revised 23 March 2017; accepted 4 April 2017; accepted article preview online 25 April 2017; advance online publication, 23 May 2017

classification.<sup>17–19</sup> Bone marrow morphology, flow cytometry and cytogenetics were centrally reviewed. National ethics committees and institutional review boards approved this study, and patients or guardians provided written informed consent. The study was performed in accordance with the Declaration of Helsinki.

## Methods

Cytogenetic data were available for 701 patients (92%) and were collected and centrally reviewed as previously described.<sup>2</sup> The descriptions of karyotypes followed the International System of Human Cytogenetic Nomenclature.<sup>20</sup> For the following analyses, we excluded patients with t(15;17)/PML-RARA-fusion gene (probability of event-free survival (EFS) 89 ± 4%, *n* = 59) due to their unique biology and treatment.

Cytogenetics, fluorescence *in situ* hybridization (FISH) and reverse transcription polymerase chain reaction (RT-PCR) analyses were performed using standard protocols with initial bone marrow or peripheral blood samples. After short-term culture for 12–72 h, metaphases of bone marrow and/or peripheral blood were prepared according to standard procedures. Fluorescence R-banding was performed as described by Göhring *et al.*<sup>21</sup> In patients with *MLL*-rearrangement (mixed-lineage leukemia, also called *KMT2A*), t(15;17)(q22;q21), t(8;21)(q22;q22) or inv(16)(p13q22)/t(16;16)(p13;q22) the molecular genetic aberrations were analyzed to confirm the cytogenetic data by FISH on interphase nuclei and by RT-PCR.<sup>21,22</sup> In few patients the confirmation of specific aberrations in cytogenetics was performed only by FISH or RT-PCR, and patients were added to the corresponding cytogenetic subgroup. In detail 16% of patients with *MLL*-rearrangement, t(15;17) or t(8;21) were confirmed by FISH only, without additional confirmation by RT-PCR. Seven percent of patients with *MLL*-rearrangement have been confirmed by RT-PCR only.

Analysis of the *FLT3* gene (FMS-Related Tyrosine Kinase 3; OMIM No: 136351; localization: 13q12) was performed in 475 patients using PCR with 5' end D4 WellRED dye-labeled reverse primers (Beckman Coulter, Krefeld, Germany). Samples were diluted in SLS (CEQ SLS, Beckman Coulter) containing a CEQ 600 size standard mixture (CEQ DNA Size Standard Kit, Beckman Coulter). The sizes of the resulting fragments were measured by capillary fragment analysis on a CEQ8000 genetic analysis system (Beckman Coulter). Wild-type and mutated alleles were assessed as proposed by Thiede *et al.*<sup>23</sup> PCR products were subsequently sequenced using the GenomeLab Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter).<sup>23–30</sup> Detailed protocols and primer sequences are available on request.

## Risk classification

Risk stratification was performed as described.<sup>31</sup> Patients with FAB M1/M2 with Auer rods or FAB M4eo or favorable cytogenetics [t(8;21)/*RUNX1-RUNX1T1* or inv(16) or t(16;16) and/or *CBFB/MYH11*] were assigned to the standard risk group. All other patients as well as standard risk patients with *FLT3*-ITD mutations or bone marrow blasts ≥ 5% on day 15 were assigned to the high-risk group.

## Treatment

Between March 2004 and April 2010, patients were treated according to the schedule of study AML-BFM 04, as summarized previously.<sup>31</sup> Allogeneic HSCT from matched sibling donors was limited to high-risk patients in 1st CR, and after a 2006 amendment, allogeneic HSCT was restricted to only high-risk patients with persistent disease after second induction (bone marrow blasts > 5%).<sup>32,33</sup> Thereafter, patients were treated according to the AML-BFM 04 protocol but not further randomized. All patients received the liposomal formulation of daunorubicin during induction therapy, and all high-risk patients received 2-chloro-2-deoxyadenosine as intensification therapy during the cytarabine/idarubicin consolidation. Furthermore only high-risk patients received high-dose cytarabine and mitoxantrone (HAM) as second induction. Randomized cranial irradiation was stopped in May 2009.<sup>33</sup>

## Definitions

The remission criteria were defined according to the Cancer and Leukemia Group B criteria at the end of intensification.<sup>34</sup> EFS was calculated as the time from diagnosis to the first event (relapse, death of any cause, failure to achieve remission or secondary malignancy) or last follow-up. Failure to achieve remission was considered an event on day 0. Overall survival (OS) was defined as the time from the date of diagnosis to the date of death

from any cause or last follow-up. Death within 42 days was considered early death.

## Cytogenetic definitions

MK was defined as either loss of at least two autosomes or one autosome and at least one structural abnormality excluding marker and ring chromosomes. Favorable cytogenetics were excluded. This definition was previously described by Breems *et al.*<sup>8</sup> and excluded patients with only one autosomal monosomy combined with a marker chromosome but no additional aberration. For further analysis, we defined a subgroup of patients who fulfilled the MK<sup>+</sup> criteria but had no involvement of monosomy 7 (referred to as MK<sup>+</sup> no <sup>−7</sup>). HK was defined as < 46 chromosomes. Patients with HK (HK<sup>+</sup>) were subdivided as those who fulfilled the criteria for both MK and HK (HK<sup>+</sup>/MK<sup>+</sup>) or HK<sup>+</sup>/MK<sup>−</sup>. CK was defined as previously described by von Neuhoff *et al.*<sup>2</sup> by three or more aberrations, including at least one structural aberration, without favorable genetics and without *MLL*-rearrangement. Unbalanced translocations were counted as one abnormality. Patients with CK (CK<sup>+</sup>) were similarly separated into subgroups according to whether they also fulfilled the criteria for MK: CK<sup>+</sup>/MK<sup>+</sup> and CK<sup>+</sup>/MK<sup>−</sup>. For further analysis, patients were analyzed regarding the number of aberrations as described by Grimwade *et al.*<sup>35</sup> Therefore, we referred to the definition of Grimwade and counted an unbalanced translocation as two abnormalities, but again excluded patients with *MLL*-rearrangement or favorable genetics.<sup>35</sup>

In addition, we analyzed patients with MK, excluding any presence of a marker or ring chromosome and patients with marker chromosomes only, but no MK.

Aberrations in chromosome 12p were counted as abnormality independent of the breakpoint or other aberrations. For this analysis, a subgroup of patients was retrospectively defined as having a very high risk of relapse depending on the following cytogenetic criteria: inv(3)(q21q26.2)/t(3;3)(q21;q26.2), t(6;9)(p23;q34), t(7;12)(q36;p13), t(5;11)(q35.3;p15), t(9;22)(q34;q11), monosomy 7, aberrations in chromosome 12p, and *MLL*-rearrangement due to t(4;11)(q21;q23), t(6;11)(q27;q23) or t(10;11)(p12;q23).

## Statistical analyses

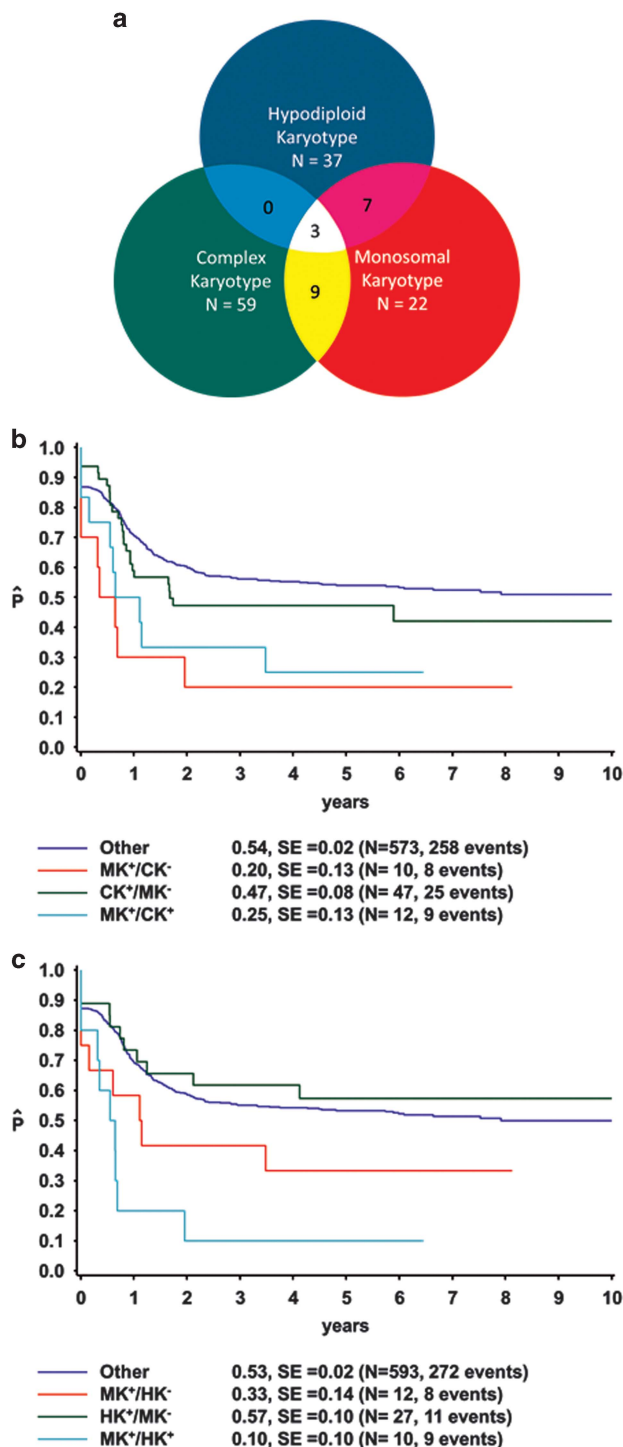
Statistical analyses were performed with SAS version 9.03 (SAS Institute, Cary, NC, USA). The median follow-up was 5.6 years. The Kaplan–Meier method was applied to estimate probabilities of survival. EFS and OS were compared with the log rank test. Construction of the cumulative incidence of relapse (CIR), the cumulative incidence of nonresponse and death in continuous complete remission (CCR) was based on the Kalbfleisch and Prentice method.<sup>36</sup> Gray's method was used to compare cumulative incidences. The Cox proportional hazards model was used for multivariate analysis of outcomes or nonresponse and relapse. The following parameters were used for multivariate analysis: cytogenetic standard risk group, MK, monosomy 7, isolated trisomy 8, *FLT3* mutation, other genetically defined high-risk factors, bone marrow day 15 and HSCT. Proportions were compared between groups using the Chi-square or Fisher's exact test. If not further specified, we refer to the remaining patients (others than patients of the analyzed subgroup) as comparator group. For further analysis and discussion, published data from study AML-BFM 98 were included.<sup>2</sup> The database lock for this analysis was set at first of July 2016.

## RESULTS

### Prognoses among cytogenetic subgroups in the AML-BFM 04 study

The 5-year EFS of all study patients with cytogenetic data (*n* = 701) was 55 ± 2% compared to 49 ± 2% in our previous study (*n* = 457, AML-BFM 98).<sup>2</sup> For detailed information regarding incidences, outcomes and initial data of cytogenetic aberrations see Supplementary Tables 1 and 2. If not otherwise specified, the following outcome data always refer to the remaining patients excluding patients with t(15;17) (others than patients of the analyzed subgroup) as comparator group.

Patients with favorable cytogenetic aberrations showed an excellent response to induction therapy with CR rates of 95% (t(8;21)(q22;q22)/*RUNX1-RUNX1T1*) and 97% (inv(16)(p13.1q22)/



**Figure 1.** (a) Schematic presentation of overlapping groups of patients with AML according to their cytogenetically defined karyotype. (b) Event-free survival of patients with complex (CK) and monosomal karyotype (MK). (c) EFS for patients with MK and hypodiploid karyotype (HK).

*CBFB-MYH11*) and significantly better survival rates (EFS  $65 \pm 6\%$ ,  $P = 0.028$  and EFS  $77 \pm 6\%$ ,  $P < 0.0001$ ) compared to the remaining patients of the study (Supplementary Table 2). However, the EFS of patients with *t*(8;21) was significantly lower compared to that of AML-BFM 98 ( $65 \pm 6\%$  vs  $84 \pm 5\%$ ,  $P = 0.021$ ) due to a temporary change in the reinduction therapy without high-dose cytarabine and mitoxantrone (HAM) in study AML-BFM 04 as described by

Creutzig *et al.* (Supplementary Table 2).<sup>37</sup> In contrast to our previous study, the prognosis in patients with an *MLL*-rearrangement was not significantly inferior to that of the remaining cohort (EFS  $54 \pm 4\%$ ,  $P = 0.91$ ) and was significantly better than that in the AML-BFM 98 cohort (EFS  $54 \pm 4\%$  vs  $34 \pm 5\%$ ,  $P = 0.0063$ ; Supplementary Tables 2 and 5). Patients with aberrations of chromosome 7q had a worse prognosis (EFS  $21 \pm 13\%$ ,  $P = 0.0073$ ) than those in the AML-BFM 98 study, but only in terms of EFS (Supplementary Table 2).

This study confirmed the poor prognosis in terms of EFS and CIR associated with aberrations in 12p (EFS  $24 \pm 9\%$ ,  $P = 0.0011$ ; CIR  $53 \pm 11\%$ ,  $P = 0.01$ ; Supplementary Table 2).

In addition, patients with trisomy 8 had a significantly poorer outcome compared to the other patients of the study, but only if this aberration was exclusive (EFS  $25 \pm 11\%$ ,  $P = 0.0091$  and OS  $42 \pm 13\%$ ,  $P = 0.011$ ). Four patients were nonresponders (25%), and seven patients relapsed (44%) (Supplementary Tables 1 and 2). On multivariate analysis, isolated trisomy 8 showed a hazard ratio (HR) of 1.98 for EFS (95% confidence interval (CI) 0.99–3.97,  $P = 0.053$ ; Table 3).

Patients with the retrospectively analyzed high-risk factors according to genotype (see cytogenetic definition) (13%) had a significantly lower survival (EFS  $33 \pm 4\%$ ,  $P < 0.0001$  and OS  $57 \pm 6\%$ ,  $P = 0.0003$ ) compared to other patients (Supplementary Table 2). CR was achieved by 75% of these patients. The predicted poor outcomes were confirmed retrospectively in the AML-BFM 98 data (EFS  $22 \pm 7\%$ ).

For the following analyses, outcome results of all patients with cytogenetic data were used to evaluate the novel high-risk criteria in detail. If not otherwise specified, the following sections always refer to the remaining patients (others than patients of the analyzed subgroup, excluding patients with *t*(15;17) as comparator group.

#### Prognosis with novel cytogenetic high-risk criteria in the study AML-BFM 04

A high level of complexity based on overlapping subgroups (MK<sup>+</sup>, CK<sup>+</sup> and HK<sup>+</sup>) was detected in our cohort (see Figure 1a; initial data in Table 1). Several patients met the definitions of more than one subgroup, and three patients fulfilled the criteria for all three definitions. Of these three patients, two experienced relapse, and one is in CCR.

#### Prognosis with monosomal karyotype

Twenty-two patients (3%) met the criteria for MK<sup>+</sup> as defined by Breems *et al.* (for details on the distribution of monosomies in patients with MK, see Figure 2a and Supplementary Table 6). MK<sup>+</sup> patients were younger (median age, 3.9 years) than the comparator group and showed significantly reduced EFS and OS compared to other patients ( $23 \pm 9\%$ ,  $P = 0.0003$  and  $35 \pm 10\%$ ,  $P < 0.0001$ , respectively; Tables 1 and 2, Figures 2b and c). Seventy-seven percent of MK<sup>+</sup> patients achieved CR after induction therapy, but the CIR was high ( $46 \pm 11\%$ ,  $P = 0.08$ ). Multivariate analysis identified MK<sup>+</sup> as an independent high-risk factor (EFS: HR 2.44, 95% CI 1.27–4.69,  $P = 0.007$  and nonresponse or relapse: HR 2.56, 95% CI 1.28–5.09,  $P = 0.007$ ; Table 3). Fourteen MK<sup>+</sup> patients were analyzed for *FLT3* mutations, and no such mutation was detected.

#### Monosomal karyotype and monosomy 7

Four MK<sup>+</sup> patients also were missing chromosome 7 (Table 1). After excluding these patients, the remaining patients (labeled MK<sup>+</sup> no 7) still experienced a poor outcome (EFS  $28 \pm 11\%$ ,  $P = 0.0081$ ; Table 2). Although 89% of them achieved CR, their risk of relapse was high (CIR  $50 \pm 13\%$ ,  $P = 0.03$ ; Table 2). Only two patients had an isolated monosomy 7. Both patients died after



**Table 1.** Initial data for monosomal, complex and hypodiploid karyotypes

	With cytogenetic data		Monosomal karyotype (MK+)		MK+ excluding -7		MK+ and complex karyotype (MK+/CK+)		MK+ and hypodiploid karyotype (MK+/HK+)		Complex karyotype		Hypodiploid karyotype	
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Patients	642	100	22	100	18	100	12	100	10	100	59	100	37	100
Age (median, years)	9	—	3.9	—	3.2	—	3.9	—	5.2	—	3.7	—	10	—
Male	334	52	6	27	3	17	3	25	3	30	30	51	20	54
Female	308	48	16	73	15	83	9	75	7	70	29	49	17	46
M0	27	4	3	14	1	6	1	8	3	30	8	14	4	11
M1/M2	247	38	6	27	5	28	4	33	2	20	20	34	27	73
M4 Eo+	64	10	—	—	—	—	—	—	—	—	—	—	—	—
M4Eo- /M5	239	37	8	36	7	39	2	17	4	40	14	24	5	13
M6	13	2	1	5	1	6	1	8	—	—	4	7	—	—
M7	43	7	4	18	4	22	4	33	1	10	13	22	1	3
Non-classified	9	1	—	—	—	—	—	—	—	—	—	—	—	—
CNS+	85	13	2	9	1	6	1	8	—	—	7	12	1	3
Organ+	170	26	7	32	5	28	2	17	3	30	17	29	6	16
WBC (median, 10 <sup>3</sup> /μl)	19 800	—	9900	—	12 590	—	9900	—	11 390	—	11 370	—	11 020	—
Hb (median, g/dl)	8.1	—	7.4	—	7.7	—	7.9	—	7.8	—	8.1	—	7.1	—
BM blasts day 15 ≥ 5%	109	17	6	27	5	28	3	25	4	40	17	29	8	22

Abbreviations: BM, bone marrow; CNS+, with CNS involvement; Eo-, without atypical eosinophils; Eo+, with atypical eosinophils; Hb, hemoglobin; Organ+, extramedullary disease apart from spleen and liver; WBC, white blood cell count.

nonresponse. Overall, the seven patients with monosomy 7 showed the expected adverse outcome (EFS  $14 \pm 13\%$ ,  $P=0.0018$ ), with a poor response to therapy (CR 29%,  $P<0.001$ ) compared to all other patients (Supplementary Table 2).

Multivariate analysis of EFS, established to distinguish MK<sup>+</sup> no -7 patients and those with monosomy 7, identified monosomy 7 as independent prognostic factors for poor survival (monosomy 7: HR 4.53; 95% CI 1.43–14.35;  $P=0.01$  and MK<sup>+</sup> no -7: HR 2.08, 95% CI 0.96–4.48,  $P=0.062$ ; Supplementary Table 3). This was further confirmed by Cox regression analysis of the incidence of nonresponse and relapse (Supplementary Table 3).

#### Monosomal and complex karyotype

Twelve patients met the criteria for MK<sup>+</sup>/CK<sup>+</sup> and had a very poor prognosis (EFS  $25 \pm 13\%$ ,  $P=0.024$  and OS  $25 \pm 13\%$ ,  $P=0.0001$ ) compared to that of other patients (Table 2). The remaining MK<sup>+</sup> patients without a CK (MK<sup>+</sup>/CK<sup>-</sup>) had a similarly unfavorable prognosis (EFS  $20 \pm 13\%$ ,  $P=0.0029$ ; Figure 1b and Table 2). The presence of CK did not change outcome of MK patients significantly (EFS MK<sup>+</sup>/CK<sup>-</sup> vs MK<sup>+</sup>/CK<sup>+</sup>,  $P=0.58$ ). Also, the poor outcome of patients with MK was not dependent on the presence of complex aberrations according to Grimwade *et al.* (Supplementary Table 4).

#### Monosomal and hypodiploid karyotype

Ten patients who fulfilled the criteria for both MK and HK showed very poor EFS ( $10 \pm 10\%$ ,  $P<0.0001$ ; Figure 1c) and a significantly increased CIR ( $70 \pm 17\%$ ,  $P<0.0001$ ; Table 2) compared to other patients. The event-free survival of MK<sup>+</sup>/HK<sup>-</sup> vs MK<sup>+</sup>/HK<sup>+</sup> was not significantly different ( $P=0.19$ ). HK<sup>+</sup> independent of MK<sup>+</sup> did not significantly affect patients' outcome (EFS  $44 \pm 8\%$ ,  $P=0.30$ ; Table 2).

#### Monosomal and marker chromosomes

Ten out of 22 patients with MK showed the presence of a marker chromosome, including one ring chromosome (45%). Patient with MK without any involvement of a marker chromosome still showed a very poor survival (EFS  $25 \pm 13\%$ ,  $P=0.007$  and OS

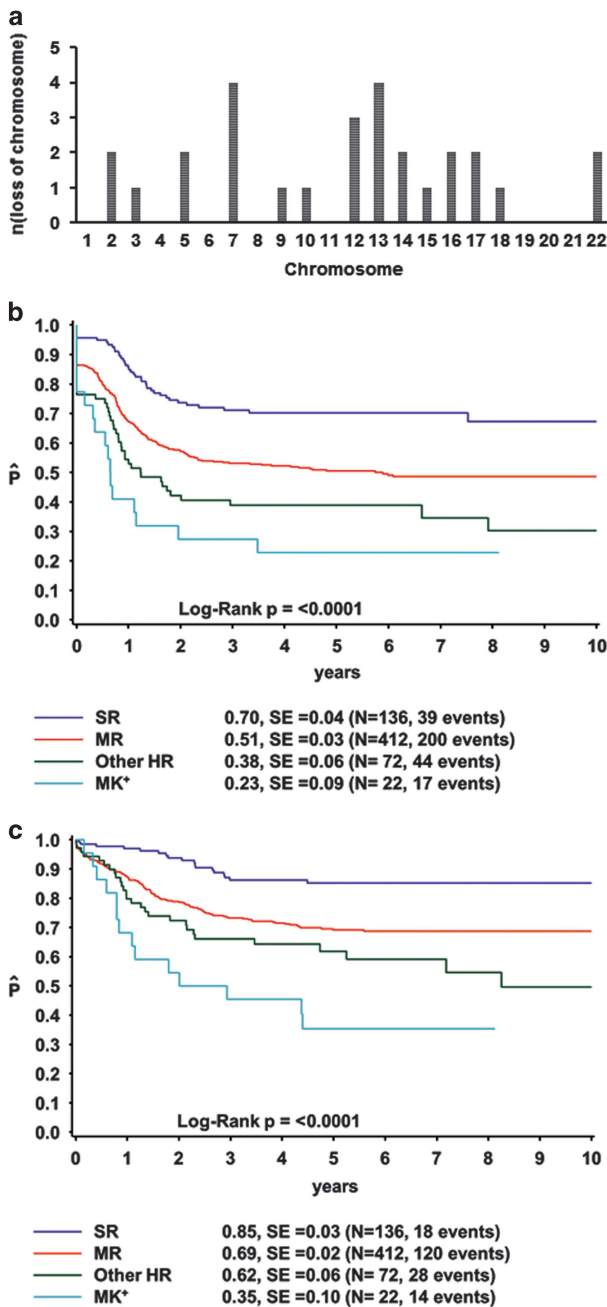
$40 \pm 15$ ,  $P=0.011$ ; Table 2). The event-free survival of patients with presence of a marker chromosome but no MK was not significantly different compared to the remaining patients of the comparator group (EFS  $45 \pm 10\%$ ,  $P=0.30$  and OS  $62 \pm 10\%$ ,  $P=0.44$ ; Table 2).

#### Prognosis with complex karyotype

Fifty-nine patients (9%) were CK<sup>+</sup> (see Table 1 for initial data), and only 3 of the 32 analyzed CK<sup>+</sup> patients had an additional *FLT3* mutation. Of note, no patient with CK<sup>+</sup> showed a monosomy 7. Survival in CK<sup>+</sup> patients did not differ from that of other patients (EFS  $43 \pm 7\%$ ,  $P=0.099$  and OS  $58 \pm 7\%$ ,  $P=0.082$ ; Table 2). After exclusion of patients with additional criteria for MK<sup>+</sup>, the remaining patients had an EFS of  $47 \pm 8\%$  ( $P=0.46$ ) and OS of  $68 \pm 7\%$  ( $P=0.91$ ; Table 2). An additional analysis showed that complex karyotypes with 2–3, 4–5 or >5 aberrations according to the definition of Grimwade *et al.* did not show significant changes in EFS, whereas patients with >5 aberrations had a reduced OS ( $P=0.0008$ ) independent of the presence of MK (Supplementary Table 4).

#### Prognosis with hypodiploid karyotype

A HK ( $n=37$ , 6%) was frequently detected in FAB M2 patients (54%) (Table 1). HK<sup>+</sup> associated with t(8;21) was characterized by loss of sex chromosomes ( $n=21$ , 57%). Overall, the outcomes among HK<sup>+</sup> patients did not differ from those of other patients (EFS  $44 \pm 8\%$ ,  $P=0.30$ ; Table 2). As described the presence of MK significantly influenced patient's outcome (EFS HK<sup>+</sup>/MK<sup>-</sup> vs HK<sup>+</sup>/MK<sup>+</sup>,  $P=0.04$ ). In patients with t(8;21), concurrent HK did not have a significant impact on prognosis (EFS  $70 \pm 10\%$  ( $n=21$  HK<sup>+</sup>) vs  $63 \pm 7\%$  ( $n=55$  HK<sup>-</sup>),  $P=0.5$ ). However, after exclusion of patients with t(8;21), the remaining HK<sup>+</sup> patients had a very poor prognosis ( $n=16$ , EFS  $9 \pm 8\%$ ,  $P<0.0001$ ; Table 2). Six of these patients did not have concurrent MK<sup>+</sup> and all had an event (EFS=0).



**Figure 2.** (a) Distribution of monosomies in patients with monosomal karyotype. *n*=number of monosomies; MK, monosomal karyotype. (b) EFS for patients with MK compared to patients with standard risk (SR), intermediate (MR) and other high-risk factors. (c) OS for patients with MK compared to patients with SR, intermediate risk and other high-risk factors. Definition of intermediate risk according to the AML-BFM 2012 protocol, defined as no favorable or high-risk criteria.

## DISCUSSION

Conflicting data from trial groups regarding the prognostic value of several cytogenetic and molecular aberrations have prevented the establishment of uniform risk-adapted therapy in pediatric AML. Accordingly, the prognostic significance of the isolated or combined occurrence of several factors such as MK, CK, HK and trisomy 8 has remained largely unclear. Our aim was to elucidate the role of potentially unfavorable risk factors in a large cohort of

pediatric AML patients. So far, no pediatric study has included a clearly defined MK as a high-risk factor. In adults with AML, MK is a well-recognized adverse prognostic factor based on several large studies demonstrating the extremely poor prognosis of these patients.<sup>8,10,12</sup> These studies further showed an association of MK with multidrug resistance activity and reported conflicting results regarding the possible benefit of HSCT.<sup>9,38–41</sup> Therefore, the best risk-adapted therapeutic approach for these high-risk patients is still under debate.

In one pediatric study, Manola *et al.* did not detect significant differences in the initial response to induction therapy in 15 out of 244 (6%) patients with MK. However, small patient numbers and a high percentage of patients categorized as having AML with MDS-related features limited their study.<sup>14</sup> Notably, in childhood MDS, MK is associated with a heterogeneous prognosis,<sup>42</sup> which is in accordance with adult studies and thus creates doubt concerning the independent prognostic value of this karyotype in MDS.<sup>43–45</sup>

Here, in a much larger cohort of pediatric AML patients we identified MK as a strong and independent predictor of high-risk AML in univariate and multivariate analysis that does not require involvement of chromosome 7. These patients showed an extremely poor EFS and OS. Consistent with studies in adults, we did not detect any *FLT3* mutations in our MK<sup>+</sup> patients, but also identified a relevant overlap between MK and CK.<sup>46,47</sup> We included the presence of a marker chromosome that may be a result of pronounced clonal instability, even though a monosomy combined with a marker chromosome often may not be a complete monosomy. However, we excluded patients with only one autosomal monosomy combined with a marker chromosome, but no additional aberration as described by Breems *et al.*<sup>8</sup> As described before in patients with MDS, 45% of our patients with MK showed the presence of a marker chromosome, which is in a range with Schanz *et al.*<sup>45</sup> Of note, an additional analysis with exclusion of patients with any involvement of a marker chromosome still showed a very poor survival (EFS *P*=0.007 and OS *P*=0.011).<sup>8</sup> The prognosis did not differ compared to the other definitions of MK in this cohort. The EFS of patients with presence of a marker chromosome but no MK was not significantly different compared to the remaining patients of the comparator group.

Patients with a CK only and no MK did not experience outcomes worse than the remaining patients. However, the better results of patients with CK in the current study must be interpreted with caution due to changes in therapy schedules. In the study AML-BFM 04, 11 CK<sup>+</sup> patients received HCST in 1st CR, whereas in the trial AML-BFM 98, only one patient underwent transplantation. This might indicate treatment-dependent variability of this finding and should be considered in the establishment of further risk definitions.

Of note, a small subgroup of patients that fulfilled the definition of MK has been described in case reports: monosomy 7 combined with ectopic virus integration site-1 aberrations and/or abnormalities in chromosome 3q26/inv3. This karyotype seems to confer a very poor prognosis and may be associated with diabetes insipidus.<sup>48–50</sup> One patient in our cohort had this karyotype. He showed an exceedingly complicated course, never achieving CR and dying only 6 months after diagnosis.

In our study cohort, we confirmed the incidence (6%) of hypodiploidy in pediatric AML and its association with t(8;21) and AML FAB M2 as reported by the pediatric Nordic Society of Pediatric Hematology and Oncology (NOPHO) study (incidence 8%).<sup>15</sup> We could not verify a male predominance or an unfavorable overall prognosis for these patients (EFS 44±8%, *P*=0.3). However, the small subgroup of patients who fulfilled criteria for MK and HK or HK without concurrent t(8;21) were identified as having a very high risk of relapse and poor survival.

**Table 2.** Treatment results in patients with monosomal, complex and hypodiploid karyotypes

	Patients		CR		SR	HR	5-years pEFS		P (Log rank) <sup>a</sup>	5-year pOS		P (Log rank) <sup>a</sup>	CIR		P (Gray) <sup>a</sup>
	n	%	n	%	%	%	%	SE		%	SE		%	SE	
Total no. of patients with cytogenetic data (excluding t(15;17))	642	100	558	87	29	71	52	2		70	2		32	2	
Monosomal karyotype <sup>b</sup> (MK+)	22	3	17	77	—	100	23	9	0.0003	35	10	< 0.0001	46	11	0.08
monosomal karyotype excluding marker chromosomes	12	2	8	67	—	100	25	13	0.007	40	15	0.011	42	16	0.29
monosomal karyotype <sup>b</sup> excluding –7	18	3	16	89	—	100	28	11	0.0081	38	12	0.001	50	13	0.03
MK+ and complex karyotype (MK <sup>+</sup> /CK <sup>+</sup> )	12	2	10	83	—	100	25	13	0.024	25	13	0.0001	42	16	0.41
MK+ without complex karyotype (MK <sup>+</sup> /CK <sup>–</sup> )	10	2	7	70	—	100	20	13	0.0029	50	16	0.084	50	18	0.07
MK+ and hypodiploid karyotype (MK <sup>+</sup> /HK <sup>+</sup> )	10	2	8	80	—	100	10	10	< 0.0001	40	16	0.0096	70	17	< 0.0001
MK+ without hypodiploid karyotype (MK <sup>+</sup> /HK <sup>–</sup> )	12	2	9	75	—	100	33	14	0.12	30	14	0.0023	25	14	0.57
Marker chromosomes without MK	26	4	24	92	22	73	45	10	0.30	62	10	0.44	44	10	0.08
Hypodiploid karyotype <sup>c</sup> (HK <sup>+</sup> )	37	6	32	87	51	49	44	8	0.30	64	8	0.38	36	8	0.44
without t(8;21)	16	2	13	81	12.5	87.5	9	8	< 0.0001	38	12	0.0002	56	13	0.003
without monosomal karyotype (HK <sup>+</sup> /MK <sup>–</sup> )	27	4	24	89	70	30	57	10	0.50	73	9	0.70	23	9	0.33
Complex karyotype <sup>d</sup> (CK <sup>+</sup> )	59	9	54	92	12	88	43	7	0.099	58	7	0.082	44	7	0.02
complex karyotype <sup>d</sup> without monosomal karyotype <sup>d</sup> (CK <sup>+</sup> /MK <sup>–</sup> )	47	7	44	94	15	85	47	8	0.46	68	7	0.91	44	8	0.03

Abbreviations: CIR, cumulative incidence of relapse; CR, complete remission; HR, high-risk treatment of AML-BFM 2004; pEFS, probability of event-free survival; OS, overall survival; SE, standard error; SR, standard risk treatment of AML-BFM 2004. <sup>a</sup>P-value indicates whether the differences are significant in comparison to other patients with cytogenetic data (excluding patients with t(15;17)). <sup>b</sup>Two or more autosomal monosomies or one autosomal monosomy with at least one structural abnormality, according to Breems et al. No favorable cytogenetics (t(15;17)(q22;q21); t(8;21)(q22;q22); inv(16)(p13q22)/t(16;16)(p13;q22)). <sup>c</sup>< 46 chromosomes. <sup>d</sup>Three or more aberrations, at least one structural aberration; without favorable genetics; without *MLL*-rearrangement.

**Table 3.** Results of multivariate analysis including MK

	EFS			NR/relapse				
	HR	95% CI		P (Chi)	HR	95% CI		P (Chi)
		LL	UL			LL	UL	
Cylogen. SR	0.56	0.38	0.81	0.003	0.60	0.41	0.89	0.011
MK	2.44	1.27	4.69	0.007	2.56	1.28	5.09	0.007
Isolated trisomy 8	1.98	0.99	3.97	0.053	2.03	0.97	4.23	0.060
<i>FLT3</i> mutation	2.14	1.47	3.12	< 0.001	2.25	1.52	3.35	< 0.001
Other gen. high-risk factors	1.47	0.91	2.38	0.111	1.59	0.97	2.60	0.064
BM day 15	2.28	1.67	3.13	< 0.001	2.51	1.81	3.48	< 0.001
HSCT	0.28	0.13	0.58	0.001	0.18	0.07	0.44	< 0.001

Abbreviations: BM, bone marrow; CI, confidence interval; HR, hazard ratio; HSCT, hematopoietic stem cell transplantation; MK, monosomal karyotype; NR, nonresponse; EFS, event-free survival; SR, standard risk; other genetically defined high-risk factors including inv(3)/t(3;3)(q21q26.2), t(6;9)(p23;q34), t(7;12)(q36;p13), t(4;11)(q21;q23), t(5;11)(q35.3;p15), t(6;11)(q27;q23), t(10;11)(p12;q23), t(9;22)(q34;q11), aberrations in 12p.

The prognostic significance of trisomy 8 in pediatric AML remains unclear. In adult AML, controversial studies have underlined the heterogeneity of this cytogenetic group.<sup>48–50</sup> In our cohort, trisomy 8 was associated with very poor survival, but only if detected as the only cytogenetic aberration. Notably, 4 of 16 patients with trisomy 8 showed *FLT3* mutations. Owing to the observed heterogeneity and conflicting data in adult AML, more pediatric studies are required to determine the prognostic impact of trisomy 8 or hypodiploidy (without t(8;21)).

In conclusion, we identified MK as a strong prognostic factor for poor outcome in pediatric AML. Isolated trisomy 8 or HK without t(8;21) are potentially predicting a dismal prognosis. In contrast, CK alone or HK did not seem to confer an adverse prognosis. Our current study protocol (AML-BFM 2012) includes cytogenetic risk factors for stratification (standard-, intermediate- and high-risk), and HSCT in 1st CR is

recommended in cytogenetically and response-defined high-risk patients. Validation of these findings in independent pediatric patient cohorts will indicate that MK+ should be included as an important, novel adverse parameter in the list of high-risk criteria.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

We are grateful to patients for participation and data managers in all hospitals and offices for their efforts in this study. We thank JE Müller for data management and Laney Weber for proofreading the manuscript with regards to the language errors. This work was supported by the Deutsche Krebshilfe e.V. and partly by grant RVO-VFN64165 from the Ministry of Health of the Czech Republic.



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